Darwinian transformation of a “scarcely nutritious fluid” into milk

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Summary

In an early challenge to an aspect of Darwin’s theory of natural selection, Jackson Mivart contended that milk could not have evolved “from a scarcely nutritious fluid from an accidentally hypertrophied cutaneous gland”. The evolutionary change from a gland secretion to milk involves an increase in calcium and protein concentrations by up to 100 and 1000 fold, respectively. Even so, the challenge, we suggest, is not just a problem of scale. An increase in the concentrations of calcium and phosphate brings an increased risk of calcification of the secretory gland since calcium phosphate is highly insoluble. In addition, two of the four constituent milk casein proteins (κ- and α_{S2}-) aggregate to produce toxic amyloid fibrils. It is proposed that both problems were solved through the co-secretion of ancestral β- and κ-caseins to form a stable amorphous aggregate of both proteins with sequestered amorphous calcium phosphate, i.e. a primordial casein micelle. Evolutionarily, a gradual increase in the concentration of casein micelles could therefore produce progressively more nutritious fluids for the neonate without endangering the reproductive potential of the mother.

Keywords

Evolution of lactation, pathological calcification, casein proteins, amyloid fibrils

Abbreviations

ACP, amorphous calcium phosphate; OCP, octacalcium phosphate; HA, hydroxyapatite; OPN, osteopontin; P_i, inorganic phosphate; PC, phosphate centre; SCPP, secreted calcium phosphate-binding phosphoprotein; SPARCL1, SPARC-like protein 1 where SPARC is a Secreted Protein, Acidic and Rich in Cysteine, also known as osteonectin; FDCSP, gene encoding follicular dendritic cell secreted peptide 1; SCPPPQ1, gene encoding the secreted calcium-binding phosphoprotein P,Q-rich-1; CSN1S1, gene encoding α_{S1}-casein; CSN1S2, gene encoding α_{S2}-casein; CSN2, gene encoding β-casein; CSN3, gene encoding κ-casein.

Introduction

Charles Darwin in chapter VII of the sixth edition of the Origin of Species (Darwin, 1872) attempted to answer some of the criticisms of his theory of natural selection, including the view of Mr St George Jackson Mivart in chapter II of The Genesis of Species (Mivart, 1871) that milk could not have evolved "from a scarcely nutritious fluid from an accidentally hypertrophied cutaneous gland". Darwin's response, of course, stressed the adaptive advantages of even small changes of composition which, accumulating over time, could produce the large change that Mr Mivart found so hard to accept.

It is now thought that the transition from proto-lacteal secretion to nutritious milk began about 250 My ago in late Permian cynodonts and continued in early Triassic mammaliaformes (Oftedal, 2002b; a). The fossil record has contributed little to our understanding of the origins of lactation. Our hypothesis for how the transition was accomplished has arisen from comparative studies, particularly of gene and protein sequences which have revealed that caseins were derived from a group of proteins with important roles in the control of biocalcification and the likelihood is that the first caseins also had such a role. According to our hypothesis, modern caseins, with the exception of κ-casein in most species, continue to have a similar, closely related, function in milk and it is this function that is the key to understanding how nutritious milks became possible.

The proto-lacteal fluid is still considered by some (Long, 1972; Blackburn *et al.*, 1989; McClellan *et al.*, 2008) to have been a low protein and low calcium cutaneous secretion, similar in composition to sweat (Sato *et al.*, 1989; Jenkinson *et al.*, 2006) or a uterine fluid (Oftedal, 2002a). Virtually all vertebrate biofluids, including milk, blood, extracellular fluid, cerebrospinal fluid, saliva, urine and synovial fluid (Giachelli & Steitz, 2000; Mazzali *et al.*, 2002) are supersaturated with respect to the mineral phase of bones and teeth, known as hydroxyapatite (HA) and also to its precursor phases amorphous calcium phosphate (ACP)

and octacalcium phosphate (OCP) (Figure 1.). They typically contain μM or high nM concentrations of the casein protein paralogue, osteopontin (OPN) and low mM concentrations of calcium. As depicted schematically in Figure 1, casein, OPN and some other secreted phosphoproteins can sequester nanoclusters of ACP and prevent the formation of OCP or HA, even though the biofluid remains supersaturated with respect to these bulk phases. Other proteins may be present such as albumin and other phosphoproteins such as fetuin (Jahnen-Dechent *et al.*, 2011). Nevertheless, compared to milk, the nutritional value of the protolacteal fluid is likely, as Jackson Mivart maintained, to have been limited.

The adaptive change anticipated by Darwin was the improved nutrition of the neonate, achieved partly through the combined elevation of calcium and phosphate concentrations and partly through the increased concentration of casein proteins in the fluid. Caseins are unfolded proteins (Syme *et al.*, 2002); some can sequester ACP (Holt, 2004) and some have a tendency to form amyloid fibrils (Farrell *et al.*, 2003; Thorn *et al.*, 2005; Thorn *et al.*, 2008). In certain fast-growing species such as the rat and rabbit, the milk calcium concentration may exceed 100 mM with the total concentration of caseins in excess of 4 mM.

Among Eutherian milks, the casein concentration varies inversely with the lactose concentration (Jenness & Holt, 1987). Physiological measurements show that milk and blood are isosmotic and that lactose synthesis draws water into milk (Rook & Wood, 1958; 1959; Linzell & Peaker, 1971a; b; Holt, 1983). Seal milks, for example, contain very little lactose; casein concentrations can be as high as 100-150 g $(kg\ H_2O)^{-1}$ and as much as half the milk volume can be fat because of the high calorific requirement of the neonate (Oftedal, 2002a; Milligan & Oftedal, 2007; Eisert & Oftedal, 2009). By contrast, in human milk virtually all the osmotic pressure comes from lactose and the casein and fat concentrations are among the lowest of any mammal. Nevertheless, in spite of large differences in total salt concentrations

among eutherian milks, human milk has levels of supersaturation with respect to mineral calcium phosphates that are very similar to those found in other milks (Holt, 1993).

The white appearance of milk and skim milk is largely due to the scattering of light by large colloidal protein particles called casein micelles (De Kruif & Holt, 2003; Dalgleish, 2011). Among Eutherian milks, casein, calcium and inorganic phosphorus (P_i) concentrations are each positively correlated because ACP is sequestered by casein in the casein micelle. Micelles are approximately spherical in shape with radii of 600 nm or less. About 80% of the particle volume is water because of the unfolded nature of the casein polypeptide chains and about 7% of the solute mass of bovine casein micelles is calcium phosphate. Each casein micelle contains ACP nanoclusters bound tightly to short phosphorylated sequences called phosphate centre (PC) sequences found in the α_{S1}-, α_{S2}- and β-caseins. The remaining casein, κ–, is partly present in the micelle surface region and somehow limits the size of the micelles. Its relatively more hydrophilic C-terminal half forms a diffuse outer region to the micelle (Holt & Dalgleish, 1986) which is removed by the action of chymosin in the first stage of cheesemaking.

There is, however, another adaptive aspect to the transition from a proto-lacteal fluid to milk which relates not to the beneficial effect on the nutrition and growth of the neonate but to the advantage to the mother of secreting a stable fluid which neither precipitates calcium phosphate nor forms toxic amyloid fibrils. Either pathology could prejudice the survival of the suckling and reduce the lifetime reproductive potential of the mother.

Our proposed transformation involves a Darwinian process that both increases the concentrations in the lacteal fluid and avoids the potential pathological consequences for the mother. It can be briefly stated as follows. The milk casein micelle has evolved to provide a means of increasing the calcium concentration of milk without causing pathological

calcification of the mammary gland. High calcium concentrations were achieved by sequestering ACP in a stable complex formed by a phosphoprotein shell. The individual caseins that evolved for this purpose probably had a similar prior function in the control of calcification in other biofluids during the period between their origin and the evolution of lactation. Their individual tendencies to form toxic amyloid fibrils needed to be suppressed even more strongly as the concentration of caseins increased to provide a more nutritious lacteal secretion. This was achieved by secreting a mixture of caseins that form an amorphous aggregate called the casein micelle rather than amyloid fibrils. Thus in the casein micelle, certain caseins act as molecular chaperones to suppress fibril formation by the other, highly amyloidogenic, caseins.

In this paper we consider in separate subsections the evolutionary origins of the caseins, their ability to sequester ACP and their tendencies to form amyloid fibrils or act as molecular chaperones. We then bring these ideas together to provide a plausible Darwinian evolutionary mechanism for the transition from a "scarcely nutritious cutaneous secretion" to highly nutritious and stable milk, allowing the mother to go safely through repeated cycles of pregnancy, lactation and mammary gland involution.

EVOLUTION OF CASEIN GENES

The secreted calcium phosphate-binding phosphoprotein (SCPP) genes were formed initially by duplication of the 5'-region of SPARCL1 (encoding SPARC-like protein 1 where SPARC is a Secreted Protein, Acidic and Rich in Cysteine, also known as osteonectin) (Kawasaki *et al.*, 2004; Kawasaki *et al.*, 2007). Iterative gene duplications subsequently created many other SCPP genes, correlated with the diversification of vertebrate mineralized tissues in the early to middle Palaeozoic era, beginning some 550 My ago (Kawasaki & Weiss, 2008; Kawasaki, 2009) or even earlier ((Delgado *et al.*, 2001; Al-Hashimi *et al.*, 2010). Among these was the

duplication and divergence from SCPPPQ1 (encoding the bone protein SCPP PQ-rich protein 1) of the ancestral CSN2 gene coding for a β-casein-like protein. According to molecular dating of the short signal peptide sequences (Jones *et al.*, 1985) and a phylogenetic analysis (Kawasaki *et al.*, 2011), this occurred before the divergence of amniotes into the synapsid and sauropsid lineages and hence happened well before the origin of lactation. In general, molecular dating of mature casein sequences has not given credible results because of extremely high rates of sequence divergence (Jones *et al.*, 1985). Accordingly, the dating of the duplication and divergence from FDCSP (encoding follicular dendritic cell-secreted peptide) of the CSN3 gene, coding for the ancestral κ-casein, is less certain. Duplication of, and divergence from, the primordial β-casein gene produced the CSN1S1 (encoding α_{S1}-casein) and CSN1S2 (encoding α_{S2}-casein) genes prior to the evolution of mammals (Lefèvre *et al.*, 2010).

CALCIUM CONCENTRATION OF MILK

A clear indication of how primordial casein helped to raise the calcium concentration in early lacteal fluid can be inferred from the variation of casein concentrations and salt composition in modern milks and from a consideration of the function of the primordial caseins prior to lactation.

Among extant eutherian species, the total calcium concentration of milk is highly correlated with the total casein concentration (Holt, 1997). Detailed analysis among a restricted range of species reveals that inter- and intra-specific variation in milk calcium largely arises because of differences in the concentration of sequestered ACP (Holt & Jenness, 1984; Holt, 1993). The sequestered ACP is in the form of nanoclusters with an equilibrium radius of 2-4 nm (Holt *et al.*, 1996; Holt *et al.*, 1998; Holt, 2004; Little & Holt, 2004; Holt *et al.*, 2009). Bound to the surface of the nanoclusters are the most highly phosphorylated PC sequences of the

caseins, a typical example being –pSer-Leu-pSer-pSer-pSer-Glu-Glu-, where pSer is a phosphoseryl residue.

Despite the large interspecific variation in the total calcium concentration of cow's milk, in the continuous phase there is an invariant ion activity product for an acidic form of ACP (Holt *et al.*, 1981; Chaplin & Lyster, 1988; Holt, 2004). Similar ion activity products have been found in goat (Holt *et al.*, 1994) and human milks (Holt, 1993). Thus, the concentrations of sequestered ACP and casein can be varied widely without affecting the degree of supersaturation of the milk.

Although nominally supersaturated with respect to the mineral phase of bones and teeth, milk, like most other biofluids, does not normally form a precipitate of ACP. Most milks contain a concentration of casein PCs in slight excess of that required to sequester the ACP. Thus, because it is more stable, sequestered ACP forms in preference to a bulk phase of ACP. Under physiological conditions, the mineral phase of bones and teeth can only form by a process of maturation from an initial phase of bulk ACP but since the initial phase cannot form, the milk is completely stable, in spite of what may be very high total concentrations of calcium and P_i.

The stability of milk can be defined by a parameter α.

$$\alpha = \frac{[P_i]_c}{\bar{f} R_P C} \qquad (1)$$

where C is the molar concentration of casein or other sequestering protein, $[P_i]_c$ is the molar concentration of sequestered inorganic phosphate, $\bar{f}$ is the average number of PCs in a mole of casein and R_P is the number of moles of P_i per mole of PCs in calcium phosphate nanocluster or micellar calcium phosphate preparations (Holt *et al.*, 1986; Holt *et al.*, 1989;

Holt *et al.*, 1996). In milk, provided $\alpha \leq 1$, ectopic or pathological calcification is suppressed, independent of the total calcium or phosphate concentration.

Caseins contain 0 - 3 PCs, depending in part on their degree of phosphorylation. The κ-caseins normally have none, a possible exception being platypus κ-casein which has a single potential PC (Lefèvre *et al.*, 2009) but it has not yet been verified to be phosphorylated in milk. The β-caseins invariably have one potential PC. Quantitative data on casein composition are sparse for eutherian species and absent for marsupials and monotremes. Reliable published results are available for human, horse, cow and goat (Miranda *et al.*, 2004), mouse (Boumahrou *et al.*, 2009) and rabbit (Baranyi *et al.*, 1995) caseins, allowing $\overline{f}$ to be computed. These data show that human and horse caseins contain a high proportion of β-casein whereas mouse casein has a high proportion of the α_{S1}- and α_{S2}-caseins. Using the literature values for the weight percentage and casein molecular masses, the casein mole fractions have been calculated and used to determine values of $\overline{f}$ (Table 1). For all but mouse, the $\overline{f}$ values are close to 1 which is the same as all extant β-caseins and hence the presumed ACP-sequestering capacity of the primordial β-casein.

Table 1 about here.

In human milk, which has one of the lowest total calcium concentrations, the serum calcium and P_i concentrations are comparable to those in many other biofluids but the $[P_i]_c$ is about 0.5 mM. In rat milk, which has one of the highest total calcium concentrations, serum concentrations are again similar to those in many other biofluids but $[P_i]_c$ is as high as 100 mM (Holt & Jenness, 1984). In a typical biofluid other than milk, the total sequestering peptide concentration is in the range of high nM to low μM so the $[P_i]_c$ is in the low μM

range. Sequestered calcium or P_i concentrations are therefore almost negligible compared to the continuous phase concentrations which are in the low mM range (Holt *et al.*, 2009).

Ectopic mineralization is fortunately rare or benign in the healthy mammary gland because the calcium is normally sequestered in a stable state and there is a stoichiometric excess of PCs. Calculations show that in late lactation or during mastitic infections, when the pH is raised, this is not always the case and therefore the milks can become unstable (Holt, 2004).

Because the extant lizard SCPPPQ1 and β-caseins have a single PC and unfolded conformation, these features were most likely conserved in the primordial β-casein. Ancestral κ-casein may also have had a single PC, like lizard FDCSP or, potentially, platypus κ-casein and it too is predicted to have the open and flexible conformation needed to sequester ACP (Holt *et al.*, 2009). What the original function of these proteins was is unknown but it is reasonable to suppose that they were involved in some aspect of the control of biomineralization, as are many other SCPPs, and were possibly expressed in other biofluids or other mineralized tissues. In short, the new nutritional function of the primordial β-casein or ancestral κ-casein in the proto-lacteal fluid was a simple adaptation of a closely related antecedent function. Indeed, the far-from-ideal proportions of certain essential amino acids in the caseins (Hambraeus & Lonnerdal, 2003) suggests that the unfolded conformation and need to sequester ACP have been important evolutionary constraints affecting casein primary structures, even to the extent of reducing their amino acid nutritional value.

Equation (1) shows that by increasing the casein concentration, the potential calcium concentration of milk could be enormously increased without enhancing the risk of ectopic calcification. However, raising the casein concentration brings with it a different, potentially pathological, problem of protein aggregation.

CASEIN AMYLOID

A broad range of human diseases arises from the failure of specific peptides or proteins to adopt, or remain in, their native functional conformation. These pathological conditions are generally referred to as protein misfolding (or protein conformational) diseases. Among the most common of these pathologies are the neurological diseases Alzheimer's and Parkinson's, and the spongiform encephalopathies, e.g. Creutzfeldt-Jakob disease. A fuller discussion is given by Chiti and Dobson (Chiti & Dobson, 2006). The largest group of misfolding diseases is associated with the conversion of specific peptides or proteins from their soluble functional states ultimately into β-sheet-containing amyloid fibrillar aggregates. Cellular toxicity is probably not associated with the fibrils themselves but mainly arises from the oligomeric or pre-fibrillar states. Another consequence of amyloid fibril formation is amyloidosis in which large quantities of a fibril-forming protein accumulate extracellularly in specific tissues, leading to major physiological dysfunction. For example, dialysis-related amyloidosis occurs in long-term haemodialysis patients from large-scale amyloid fibril accumulation in muscle of the protein β2-microglobulin (Gejyo *et al.*, 1985).

Amyloidosis arises from the extracellular aggregation of proteins via a partially folded intermediate state to form amyloid fibrils that adopt a highly ordered cross-β-sheet structure (Chiti & Dobson, 2006). Unfolded proteins that adopt little or no ordered structure, e.g. α-synuclein and amyloid β which are involved with Parkinson's and Alzheimer's diseases respectively, are particularly prone to amyloid fibril formation. Amyloidoses are rare or benign in the non-cancerous mammary gland in spite of the presence of high concentrations of extracellular, unfolded casein proteins and repeated cycles of tissue remodelling, when the potential for fibril formation would be high due to the associated stresses being placed upon the proteins. Mineralized amyloid stones (*corpora amylacea*) containing some casein

peptides are formed in the mammary gland in late lactation and during involution (Niewold *et al.*, 1999) but they seldom affect the efficiency of lactation.

According to Goldschmidt *et al.* (Goldschmidt *et al.*, 2010), the primary structure of most proteins contains at least one potential amyloid-forming sub-sequence. However, a number of mechanisms have evolved to protect globular proteins against aggregation including burying the sub-sequence in a folded domain and the deployment of folding assistants such as molecular chaperones. Many molecular chaperones (e.g. the small heat-shock proteins (sHsps)) have extensive regions that are disordered or unfolded in conformation (Bagneris *et al.*, 2009; Jehle *et al.*, 2010; Laganowsky *et al.*, 2010) which facilitates their interaction with, and stabilisation of, a diversity of target proteins during chaperone action. Paradoxically, it also allows even molecular chaperones to form amyloid fibrils under slightly destabilizing conditions (Meehan *et al.*, 2004; Meehan *et al.*, 2007). In unfolded proteins such as caseins, the option to bury amyloidogenic sub-sequences within their own structure is absent but an alternative is to form an amorphous, non-toxic, aggregate (i.e. the casein micelle) to isolate and immobilize them (Dobson, 1999).

Purified bovine κ- or α_{S2}- caseins readily form amyloid fibrils at physiological pH (Farrell *et al.*, 2003; Thorn *et al.*, 2005; Léonil *et al.*, 2008; Thorn *et al.*, 2008). The rate of fibril formation by κ-casein is inhibited by β-, α_{S1}- and, to a lesser extent, α_{S2}-casein (Thorn *et al.*, 2005; Treweek *et al.*, 2011) but only α_{S1}-casein is an effective inhibitor of fibril formation by α_{S2}-casein (Thorn *et al.*, 2008). Complete inhibition of κ-and α_{S2}-casein fibrillogenesis by β- and α_{S1}-casein respectively requires a 2-4-fold molar excess of the inhibitor casein in each case. The inhibitory action of the caseins can be likened to the action of molecular chaperones in limiting the aggregation of partially folded proteins by forming an amorphous

aggregate rather than refractory amyloid fibrils (Bhattacharyya & Das, 1999; Morgan *et al.*, 2005).

The potential of casein and related proteins to form amyloid fibrils was investigated using a recent algorithm which threads a sub-sequence into a known cross-β-sheet structure to determine whether it is energetically and sterically compatible with the amyloid fibril structure (Thompson *et al.*, 2006; Goldschmidt *et al.*, 2010) according to the change in Rosetta energy. The structural template was the cross β-sheet fibril structure formed by the hexapeptide NNQQNY and each sub-sequence of six residues within the casein protein that does not contain a Pro residue was tested. Compatible sub-sequences are called zippers because they are predicted to nucleate the growth of amyloid fibrils. Predicted zipper sequences such as those shown in Figure 2 were manually edited as follows. Hexapeptide sub-sequences containing Cys were excluded since virtually all Cys residues in caseins form disulphide bonds (Bouguyon *et al.*, 2006). Sub-sequences containing sites of phosphorylation and glycosylation were frequently predicted to be zippers but were also excluded because of these post-translational modifications. Figure 2 shows the unedited predictions for bovine caseins in the form of a histogram of the change in Rosetta energy for hexapeptide sub-sequences versus the position in the whole sequence of the first residue of the hexapeptide. The sub-sequences considered most capable of nucleating the growth of an amyloid fibril, the so-called zipper sequences, have a calculated change in Rosetta energy of -23 kcal mol^{-1} or less. Values for the percentage of these predicted amyloid zipper sub-sequences for selected species are given in Table 1. Lizard FDCSP and SCPPPQ1 contained 0 and 6 % zipper sub-sequences, respectively. In caseins, the values ranged from zero in monotreme κ-caseins to nearly half of the mature sequence of rat α_{S1}-casein. The latter is comparable to the fraction found in highly amyloidogenic proteins like α-synuclein. Among the κ-caseins, bovine and caprine sequences had the highest fractions of zipper sub-sequences. In general, eutherian

caseins contained higher fractions of fibrillogenic sequence than monotreme or marsupial caseins. Individual values for the percentage zipper sub-sequences were averaged over the mole fractions in each of the five eutherian species for which there are reliable data. The average fraction of zipper sub-sequences was close to 20% for whole casein from four species but for human casein, the average was only half this value (Table 1). Not all predicted zipper sub-sequences will form amyloid fibrils at a measurable rate but the evidence from bovine caseins, the only species studied to date, is that κ- and α_{S2}-caseins, the two caseins with the highest proportion of zipper sub-sequences (Figure 2), are also the ones in which fibril formation most readily occurs (Thorn *et al.*, 2005; Ecroyd *et al.*, 2008; Thorn *et al.*, 2008).

In summary, high concentrations of casein and hence high concentrations of sequestered ACP, can only be achieved if there is effective suppression of the tendency for fibril formation by the individual caseins. In the cow, this has been achieved by having a 2-4-fold molar excess of β- and α_{S1}-caseins acting as molecular chaperones to suppress the highly amyloidogenic nature of the κ- and α_{S2}-caseins.

CASEIN MICELLES

Despite the ready tendency of κ- and α_{S2}-caseins to form amyloid fibrils, they seldom do so *in vivo* but instead are components of the amorphous casein micelle. This is illustrated schematically in Figure 3. In pathway 1 the κ- and α_{S2}-caseins can form amyloid fibrils but in pathway 2 the additional presence of the molecular chaperones β- and α_{S1}-caseins prevents this and an amorphous aggregate results, known as the casein micelle. The casein micelle is therefore an example of a self-associating and self-regulating complex to prevent the formation of refractory and potentially cytotoxic fibrillar aggregates.

Our contention is that in a mixture of caseins, each of which may be capable of forming amyloid fibrils in isolation, the preferred aggregation pathway is one that results in the casein micelle through many alternative and nearly equivalent interactions. According to Cubellis et al., (Cubellis *et al.*, 2005), proteins with a predominance of the poly-L-proline conformation and a tendency to aggregate readily form extended H-bonded backbone-to-backbone linkages resulting in mucus, slimes and gels rather than compact aggregates. The sequence divergence of the casein group may therefore have allowed a high percentage of amyloid zipper sub-sequences to evolve because of the effective way in which they could be isolated and immobilized in the casein micelle via chaperone action, to prevent the formation of fibrillar species.

DISCUSSION

The guiding principle applied to this problem is that any increase in the nutritional value of an initial "scarcely nutritious fluid" should not be at the expense of the lifetime reproductive success of the mother.

Equation (1) shows how the calcium concentration of a biofluid can be increased without enhancing the risk of biocalcification. Thus, for a protein at a molar concentration C with $\overline{f}$ PCs, the maximum concentration of sequestered P_i is given by $R_P.C.\overline{f}$. In calcium phosphate nanoclusters formed by casein phosphopeptides R_P is constant and equal to 6.5 (Little & Holt, 2004). Assuming that this ratio does not alter, an increase in the concentration of sequestered calcium or P_i can be achieved either by increasing $\overline{f}$ or C.

The first casein-like protein was either the ancestral β-casein related to SCPPPQ1 or the ancestral κ-casein derived from FDCSP, both of which are predicted to have been unfolded proteins with a single PC near their N-terminus. Which protein came first is unclear but to a degree this is immaterial since they both have $\overline{f}$ = 1 (Table 1). The ability to sequester ACP

was already established and effected by other SCPPs such as OPN and, perhaps also, by lizard SCPPPQ1 and FDCSP, in the control of mineralization and the stabilization of biofluids. The concentrations of sequestered calcium and P_i could be increased by increasing the concentration of β- and/or κ-casein (i.e. *C* in equation (1)). Negating this is that β-casein on its own readily forms indefinitely large aggregates and κ-casein on its own readily forms amyloid fibrils. Both of these potential problems were overcome by co-secretion to form the first primitive casein micelle. In effect, the primitive casein micelle allowed the protolacteal fluid to become nutritious milk without endangering the reproductive potential of the mother through pathological amyloidosis or calcification of the mammary gland. Moreover, this transition could be accomplished by the simple expedients of increasing the concentration of secreted casein and by secreting a mixture of interacting caseins that inhibited the formation of casein amyloid fibrils.

The option to increase the concentration of sequestered calcium and P_i by increasing the value of $\overline{f}$ has been utilized to only a limited degree among eutherian species (Table 1). Some of the casein genes that have evolved from the CSN2 gene more recently encode caseins with as many as three PCs (e.g. bovine CSN1S2 encoding α_{S2}-casein (Table 1)); even larger values of $\overline{f}$ are found among some non-casein SCPPs (Holt *et al.*, 2009). The largest value of $\overline{f}$ in Table 1 for whole casein is 1.48 for mouse milk. In eutherian and marsupial milks, the value of $\overline{f}$ has been reduced by the loss of the (potential) PC from κ-casein. Other modulators of $\overline{f}$ include exon skipping events and incomplete phosphorylation of potential PCs.

Our hypothesis for the transformation of the protolacteal fluid incorporates classical Darwinian ideas on the gradual nature of evolutionary change brought about by the

adaptation of established mechanisms to new purposes. Thus, we propose that any increase of calcium and casein concentrations in a lacteal secretion, no matter how small, can be adaptive provided there are no negative consequences for the mother. The potential negative consequences for the mother are either calcification or amyloidosis of the mammary gland as a result of the huge increases in calcium and phosphoprotein concentrations in most milks compared to blood, urine, saliva and many other biofluids. The mechanism of stabilisation of milk against calcification by sequestration of ACP (Figure 1) is proposed to be essentially the same as in these other biofluids but differs profoundly in degree. The mechanism for guarding against the formation of amyloid fibrils by promoting the alternative formation of an amorphous aggregate is also found elsewhere in biology, notably in the control of protein misfolding by molecular chaperones such as the small heat-shock proteins (Rekas *et al.*, 2004; Ecroyd & Carver, 2009). In milk, the caseins themselves act as molecular chaperones by providing many alternative but largely equivalent intermolecular interactions leading to the formation of the amorphous casein micelle rather than casein amyloid.

In summary, we have provided a comment on how the transformation of a "scarcely nutritious" fluid into milk proceeded by increasing the concentrations of important nutrients while avoiding the dangers of pathological mineralization and toxic fibril formation within the mammary gland (Figures 1 and 3). Jackson Mivart's objection to a Darwinian evolutionary mechanism does not appear to have been valid even with the additional considerations we have discussed here of potential amyloidosis and calcification of the mammary gland.

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FIGURE LEGENDS

Legend to Fig. 1. Control of calcium phosphate precipitation from biological fluids through sequestration of ACP by certain secreted phosphoproteins. The solid line traces the sequence of changes in bulk solid phase structures and the change of the system free energy (ΔG) following the formation of nuclei in a supersaturated solution of calcium phosphate in the absence of a phosphoprotein. Initial bulk phases are amorphous (ACP-1 and the slightly more stable ACP-2) and are typically succeeded by poorly crystalline octacalcium phosphate (OCP) and apatite-like phases culminating in the most thermodynamically stable (lowest free energy) form, hydroxyapatite (HA) (Meyer & Eanes, 1978; van Kemenade & Bruyn, 1987; Christoffersen *et al.*, 1990). In the presence of a molar excess of a competent ACP-sequestering phosphoprotein, no bulk phases are formed. Instead, a stable complex is formed by the phosphoprotein with the ACP. The complex is more stable than ACP-1 so that neither a bulk phase of ACP-2 nor any of its successor bulk phases can ever form and the solution remains stable indefinitely.

Legend to Fig. 2. Amyloid nucleating (zipper) sequence predictions (Goldschmidt *et al.*, 2010) for the four bovine caseins. The predictions are made on a protein sequence which includes the signal peptide, is unphosphorylated, unglycosylated and has only reduced Cys residues. The y axis in the histogram is the change of Rosetta energy for a hexapeptide forming a hypothetical amyloid fibril-like crystal. The x axis is the position in the whole amino acid sequence of the N-terminal residue of each possible hexapeptide. Thus position 1 in the histogram gives the calculated change in Rosetta energy for the hexapeptide with the sequence 1-6 of the whole protein, position 2 is for the sequence 2-7 and so on until the whole sequence has been covered. The potential hexapeptides considered most likely to nucleate the growth of an amyloid fibril give a reduction in the calculated Rosetta energy of 23 kcal mol^{-1} or more. These hexapeptides are the predicted zipper sequences which are

represented by histogram bars in warmer colours (red, orange brown). Less likely amyloid-forming hexapeptides are given cooler colours (blue, green, yellow). If the change in Rosetta energy for a given hexapeptide is positive or zero, no amyloid-like structure is predicted to be possible and so no bar appears in the histogram at its position. The propensity to form amyloid fibrils is reduced by signal peptide cleavage, phosphorylation, glycosylation and disulphide bridge formation and these factors are taken into account in calculating the percentage of zipper sequences given in Table 1.

Legend to Fig. 3. Control of casein amyloid fibril formation by a molecular chaperone-type effect. Although all four caseins contain amyloid fibril zipper sub-sequences (Fig. 2), κ- and α_{S2}-caseins are the most amyloidogenic and either of these proteins, on its own, readily forms highly structured amyloid fibrils under physiological conditions (Farrell *et al.*, 2003; Thorn *et al.*, 2005; Léonil *et al.*, 2008; Thorn *et al.*, 2008). 2. In a mixture of the four caseins, however, the α_{S1}- and β-caseins prevent the other two caseins from forming fibrils by acting as molecular chaperones (Bhattacharyya & Das, 1999; Morgan *et al.*, 2005; Treweek *et al.*, 2011). Together with sequestered ACP, the mixture of all four caseins preferentially forms an amorphous stable aggregate known as the casein micelle.

TABLE LEGEND

Table 1. Amyloid zipper residues (Goldschmidt *et al.*, 2010) expressed as a percentage of the total mature protein sequence length (%Z) and the number of PCs per mole ($\overline{f}$) in mature caseins and related peptides, after complete phosphorylation, glycosylation and disulphide bond formation.

Table 1. Amyloid zipper residues (Goldschmidt *et al.*, 2010) expressed as a percentage of the total mature protein sequence length (%Z) and the number of PCs per mole ($\overline{f}$) in mature caseins and related peptides, after complete phosphorylation, glycosylation and disulphide bond formation.

		FDCSP	**SCPPPQ1**	**-**	**-**	**-**	**Number average**
Lizard	%Z	0.0	6.0	-	-	-	
	$\overline{f}$	1	1				
Monotremes*		**CSN3**	**CSN2A**	**CSN1S1**	**CSN2B**		
Echidna	%Z	0.0	15.2	4.8	12.0		
	$\overline{f}$	0	1	3	3		
Platypus	%Z	0.0	10.3	3.6	4.3		
	$\overline{f}$	1	2	3	1		
Marsupials			**CSN2**				
Bushtail possum	%Z	13.3	11.5	14.6			
	$\overline{f}$	0	0	2			
Short-tailed opossum	%Z	3.8	18.5	11.3			
	$\overline{f}$	0	1	2			
Tamar wallaby	%Z	-	9.4	18.5			
	$\overline{f}$	-	0	2			
Eutherians					**CSN1S2A**	**CSN1S2B**	
Human	%Z	11.8	6.6	22.5	-	-	10.1
	$\overline{f}$	0	1	2			0.94
Horse	%Z	10.2	17.8	22.5	22.4	-	18.5
	$\overline{f}$	0	1	1	2		0.99
Cow	%Z	33.7	17.7	20.6	29.5	-	22.5
	$\overline{f}$	0	1	1	3		1.08
Goat	%Z	24.4	21.3	12.6	19.2		19.4
	$\overline{f}$	0	1	1	3		1.08
Rabbit	%Z	3.8	20.9	9.5	33.1		19.8
	$\overline{f}$	0	1	1	1		0.90
Mouse	%Z	9.0	20.3	30.8	17.2	16.4	19.5
	$\overline{f}$	0	1	2	2	1	1.48
Rat	%Z	7.6	31.9	49.0	19.5	29.2	-
	$\overline{f}$	0	1	2	3	0	-

* Eutherian CSN3, CSN2, CSN1S1and CSN1S2 encode κ-, β-, α_{S1}- and α_{S2}-caseins, respectively. Monotreme CSN2A is orthologous to Eutherian CSN2 but the CSN2B gene appears to be a fusion of parts of CSN2- and CSN1S2-like genes (Lefèvre *et al.*, 2010).

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